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Breast cancer cell survival signal is affected by bergapten combined with an ultraviolet irradiation

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ABSTRACT

In this study we have reported that bergapten (B) and bergapten plus UV (PUVA) are able to significantly affect MCF-7, ZR-75 and SKBR-3 breast cancer cell proliferations.

B induced a lowering of PI3K/AKT survival signal in MCF-7 cells even in presence of IGF-I stimulation. Furthermore, B and in a higher extent, PUVA up-regulated the p53 mRNA and the protein content. An increased co-association between p21 WAF and proliferating cell nuclear antigen (PCNA) has been observed in PUVA-treated MCF-7 cells, thus inhibiting DNA replication. These results highlight how B, and its photoactivated compound, exert antiproliferative effects and induce apoptotic responses in breast cancer cells.

1. Introduction

Psoralen (furocumarins) plus UVA (ultraviolet A) (320–400 nm radiation PUVA) is a widely effective therapy for psoriasis, vitiligo and other skin diseases [1–3]. Besides, the combination of psoralen and ultraviolet A radiation resulted to reduce keratinocyte and lymphocyte proliferations through a decreased secretion of cytokines. The same effects have been alternatively utilized for the treatment of human lymphoma and of autoimmune diseases through the extracorporeal photochemotherapy (ECP) [4,5].

Evidence has been provided that these photoactive compounds can interact with cellular macromolecules and can interfere with DNA synthesis, as reported in many cell types including fibroblast, keratinocytes, melanocytes or leukocytes [6–10].

Low doses of PUVA induced growth inhibition without affecting cell viability [8], while higher doses also induced apoptosis [11,12]. These compounds are currently used in the treatment of psoriasis, cutaneous T cell lymphomas and other epithelial diseases, as previously reported, while there are only few reports about the effect of PUVA on tumoral cells.

Recently, our paper investigated the pro-apoptotic effects induced by high dose of bergapten (B) (or methoxypsoralen: 5-MOP), in the absence of UV rays, in human breast cancer cells [13].

In this study, we have examined the effectiveness of photoactivated B, at low doses, on cell proliferation, utilizing three different breast cancer cell lines (MCF-7, SKBR-3 and ZR-75). In addition, the influence of drug treatment on the signalling molecules, which play a critical role in the maintenance of cell survival and apoptosis, has been evaluated in MCF-7 cells.

2. Materials and methods

2.1. Cell culture

MCF-7, SKBR-3 and ZR-75 breast cancer cells were cultured in DMEM medium supplemented with 7.5% FCS, 1% penicillin/streptomycin and 1% glutamine. Sub confluent cell cultures, synchronized for 24 h in DMEM without phenol red and serum (PRF-SFM-DMEM), were used for all experiments.

Abbreviations: UV, ultraviolet light; B, bergapten; PUVA, psoralen + ultraviolet light A; PI-3K, phosphatidylinositol-3-kinase; IGF-I, insulin-like growth factor; 5-MOP, 5-methoxypsoralen; PCNA, proliferating cell nuclear antigen

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2.2. Growth assay

All cells were seeded (100 000 cells/well) in a complete DMEM medium in six-well plates and become attached to the bottom of the well overnight. On the second day, the medium was changed and shifted for 24 h with serum-free medium. At the end of this time, the cells were treated and incubated with B (4 μM, 6 μM) for 4 h. After this incubation the cells were irradiated for 20, 40 and 60 min with UVA (365 nm) and then stowed in the incubator at 37 °C for 20 h (one set) and for 44 h (second set). At the end of this period the cells were counted in a haemocytometer by trypan blue exclusion. The results were expressed as percentage of the controls, determined by standardizing untreated cells to 100%.

2.3. Western blotting and immunoprecipitation

Cytoplasmatic and nuclear fractions, as well as total protein extracts were obtained as previously described [14]. Proteins were resolved on an 8% sodium dodecyl sulfate–polyacrylamide gel, transferred to a nitrocellulose membrane (Amersham Bioscience, Milan, Italy), and probed overnight at 4 °C with the antibody indicated in the figure legends.

Anti-p21 waf, anti-proliferating cell nuclear antigen (PCNA), anti-p53, anti-β-actin, anti-p85, anti-GAP-DH, anti-Lamin B, anti-mouse IgG and Protein A/G-agarose plus were from Santa Cruz Biotechnology. Anti-phospho-AKT (Ser 473), anti p-AKT, anti-phospho p-Bcl2 (Ser 70), anti-caspase 9 pAbs from Cell Signaling Technology.

2.4. Transfections

Transient transfection experiments were performed using plasmid pcDNA3-c-Akt encoding for AKT protein and plasmid pcDNA3 empty vector. Cells were transfected in SFM using FuGENE6 according to the manufacture's instructions with a mixture containing 0.5 μg/well of the plasmid. 24 h after the transfection the cells were treated for 24 h with B alone or in combination with UV at 40 min. At the end of this incubation the cells were collected and lysed for Western blot analysis or utilized for cell counting.

2.5. Phosphatidylinositol-3-kinase activity

Phosphatidylinositol-3-kinase (PI-3K) activity associated with p85, was assessed by standard protocol provided by the manufacturer of the p85 antibody (Upstate Biotechnology). Briefly, cell lysates of MCF-7 cells treated or untreated with B and PUVA were immunoprecipitated with anti-p85 and with Protein A/G agarose. The presence of PI3K activity in immunoprecipitates was determined by incubating the beads with reaction buffer containing 10 mM HEPES (pH 7.4), 10 mM $MgCl_2$, 50 μM ATP, 20 μCi [γ^{32}-P] ATP, and 10 μg of L-α-phosphatidylinositol-4,5-bis phosphate for 20 min at 37 °C. The reactions were stopped by adding 1 M HCl. Phospholipids were extracted with $CHCl_3$/methanol (1:1, v/v) and the labelled products of the kinase reaction, the PI phosphates, then were spotted onto trans-1,2-diaminocyclohexane-*N,N,N′,N′*-tetra acetic acid – treated silica gel 60 thin layer chromatography (TLC) plates. Radioactive spots were visualized by autoradiography [14].

2.6. RT-PCR assay

Total cellular RNA was extracted from MCF-7 cells using TRIAZOL reagent (Invitrogen, Carlobad, CA). Reverse transcription was done using RETRO script kit (AMBION, Austin, TX).

Primers sequences include: p53 forward: 5′-CTT CCT GAA AAC AAC GTT CTG TCC-3′, and reverse: 5′-CCA GAC CAT CGC TAT CTG AGC A-3′; rRNA 36B4 forward: 5′-CTCAACATCTCCCCCTTCTC-3′, and reverse: 5′-CAAATCCCATATCCTCGTCC-3′.

2.7. DNA ladder formation

Cellular DNA was isolated by following the procedure previously described [14]. The DNA fragments were resolved by electrophoresis at 75 V on 1% agarose gel impregnated with ethidium bromide, detected by UV transillumination, and photographed.

2.8. Statistical analysis

Each data point represents the mean ± S.D. of at least four experiments. The data were analyzed by analysis of variance using the STATPAC computer program.

3. Results

3.1. Bergapten and PUVA affect breast cancer cells survival

Firstly, we tested the effect of B and UV on MCF-7, SKBR-3 and ZR-75 breast cancer cell proliferations. We observed that single treatment with B after 24 h affects cell survival prevalently in MCF-7 cells whereas more consistent effects were observed under the combined treatment (PUVA) in all the cell lines tested. The prolonged treatments up to 48 hours showed the greatest antiproliferative responses in the three breast cancer cell lines (Fig. 1). Lower doses of the drug did not induce significant changes in all the cell lines tested (data not shown).

Since MCF-7 cells resulted to be more responsive to psoralen treatment, we investigated the effect of the drug and UV on the PI-3K/AKT pathway, mainly involved in the regulation of cell survival. B alone or UVA were able to down-regulate the PI3K activity, while their combination (5-MOP + UVA) did not further decrease the enzymatic activity (Supplementary Fig. 1A). Besides, either B or UV irradiation were able to down-regulate phospho-AKT levels (Ser 473) (Supplementary Fig. 1B).

The same signalling molecule was evaluated in MCF-7 under IGF-I (insulin-like growth factor) – cell stimulated condition, since this growth factor represents a key nutritional element for breast cancer cell proliferation as largely well documented [15–17]. It is worth to observe how B, both in a single treatment and in combination with UV, is able to counteract the stimulatory effect induced by IGF-I (100 ng/ml) on the phospho-AKT (Supplementary Fig. 1C).

3.2. Bergapten and PUVA treatments induce p53 and p21 WAF expression in MCF-7cells

Our recent work demonstrated that B, particularly at higher doses without UV, is able to up-regulate p53 and to transactivate p53 gene promoter [13]. On the basis of these results we wanted to assess whether lower doses of B in the presence of UV light (at different time of cell exposure) can modulate p53, p21 and phospho-Bcl-2 expression in MCF-7 cells. A representative Western blot in Fig. 2A reveals that B at both 4 and 6 μM induces an increased expression of p53 and p21 WAF which resulted more up-regulated under the combined treatment: PUVA rays; however, under B 6 μM + 60 min UV the augment is less pronounced. In this last condition the cells are undergone to consistent stress stimuli that might in turn affect the p53 and p21 WAF protein levels.

On the contrary, the phospho-Bcl-2 expression, a p53-linked protein, resulted to be down-regulated and barely detectable under the combined treatment B plus UV (Fig. 2A).

Since p53 is involved in the induction of apoptosis, next we studied the expression of caspase-9 and the pattern of DNA ladder

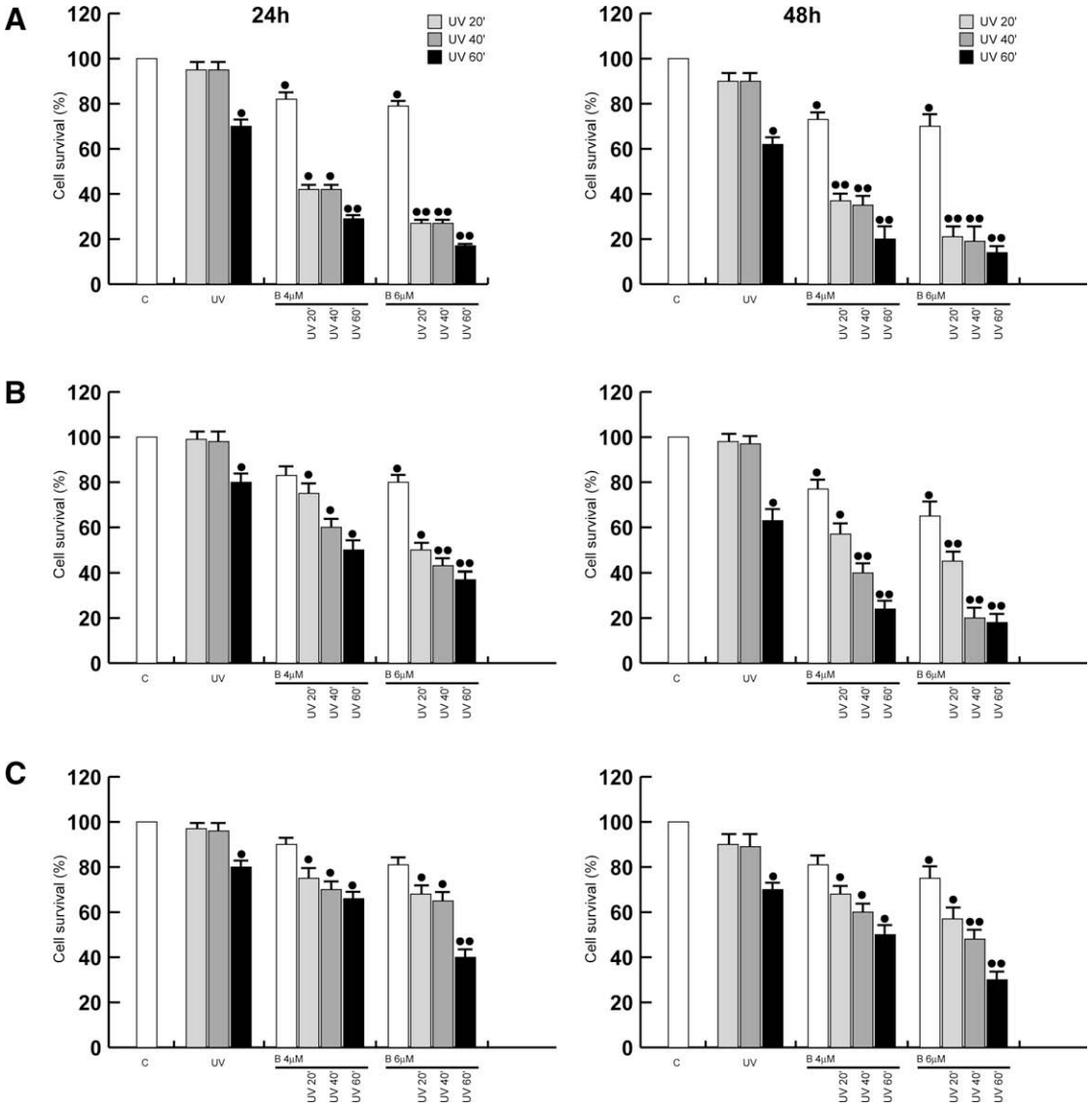

Fig. 1. Cell viability of MCF-7, SKBR-3 and ZR-75 cells after bergapten and bergapten + UVA (PUVA) treatments. (A) MCF-7, (B) SKBR-3 and (C) ZR-75 breast cancer cells seeded in six-well plates (100 000 cells/well) were treated for 24 and 48 h with 4 and 6 μM of bergapten or irradiated with UVA in a single schedule for 20, 40 and 60 min (′). Half of these plates were undergone to PUVA treatment. The values are expressed as percentage of the control, determined by standardizing untreated cells to 100%. C: control; B: bergapten. Triplicate results are expressed as mean ± S.D. ($n = 4$). ●$P < 0.05$; ●●$P < 0.01$ as compared to untreated control cells.

in MCF-7 cells. Our results showed that psoralen alone, at the highest concentration used (B6 μM), induces the cleavage of caspase-9, this event was more consistent under PUVA (B6 μM + UV 40 and 60 min) (Fig. 2B). Furthermore, as evidenced in Fig. 2C, PUVA treatment resulted in the appearance of DNA ladder.

3.3. Bergapten facilitates the nuclear translocation of the p53 and up-regulates its gene transcription

Considering that B is able to up-regulate p-53 protein levels, we checked if the psoralen could also affect the intracellular compartmentalization of the protein influencing the p53 functional activity.

As reported in the Supplementary Fig. 2A, a nuclear p53 increase was induced by B 4 and 6 μM a similar effect was reproduced by PUVA treatment.

Subsequently, to investigate whether B may influence p53 gene transcription, we performed RT-PCR in MCF-7 treated with the drug (6 μM) with or without UV rays given for 20 and 40 min. The results showed that B alone stimulates p53 gene transcription which appears more increased by PUVA (Supplementary Fig. 2B).

3.4. p21 WAF protein co-immunoprecipitates with PCNA in MCF-7 cells following PUVA. The overexpression of AKT rescues cell survival

We carried out immunoprecipitation experiments to determine whether the PUVA-induced p21 WAF might serve to recruit the PCNA, a key factor necessary for DNA polymerase δ function.

Endogenous p21 WAF protein from MCF-7 cells was immunoprecipitated with anti-p21 WAF antibody and then it was probed with anti-PCNA antibody (Fig. 3A). Only PUVA treatment in MCF-7 cells leads to an increased co-association between p21 and PCNA with respect to controls, thus resulting in inhibition of DNA replication (Fig. 3A).

Therefore, we investigated if the transient over-expression of pro-survival p-AKT protein in MCF-7 cells might affect the degree of co-association of PCNA with p21 WAF (Fig. 3B).

To this aim, we transiently transfected MCF-7 cells with plasmid vector pcDNA3-c-AKT encoding for the AKT protein and then

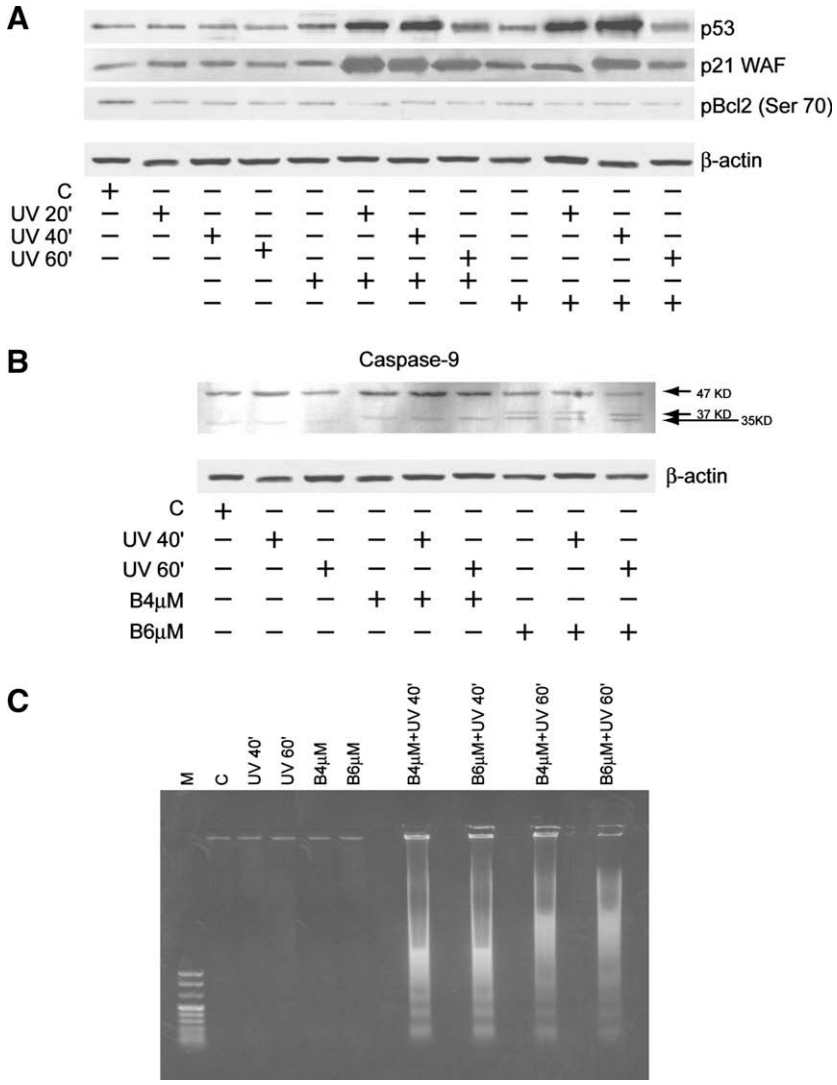

Fig. 2. Bergapten up-regulates the expression of p53, p21, p-Bcl2 while PUVA induces apoptotic events. (A) MCF-7 cells were treated for 24 h with bergapten (B) alone and UVA as indicated in terms of minutes. (B) Western blot analysis of caspase-9 in MCF-7 cells treated for 24 h with bergapten (B) and UVA as indicated. β-Actin was used as loading control. Results are representative of three independent experiments. (C) DNA ladder in MCF-7 cells. M: DNA marker; C: control.

we treated the cells with B with or without UV at 40 min (Fig. 3B). In MCF-7 cells over expressing p-AKT, as confirmed by total pAKT levels, both p21 and PCNA did not associate, while only a weak band was observed in PUVA–treated MCF-7 cells (Fig. 3B). In cells over expressing p-AKT, the levels of p21 in the immunoprecipitated samples were lower than those ones observed in MCF-7 cells.

In agreement with these last data, in MCF-7 cells the over expression of AKT protein is able to better recover cell proliferation in the presence of drug treatment compared to the results shown in Fig. 1 and obtained in MCF-7 wild type cells (Fig. 3C). Similar data were observed in the other breast cancer cells (data not shown).

4. Discussion

In this study, the effect of B, alone and in combination with UV light, has been examined on the cellular growth of breast tumoral cells.

Our data have demonstrated that treatment of MCF-7, SKBR-3 and ZR-75 breast cancer cells with B and, in a higher extent, with PUVA, has determined a lowering of cell proliferation.

The best effect has been obtained in MCF-7 cells, responsive also to the single drug treatment. This epithelial breast cancer derived MCF-7 cell line is one of the most frequent model systems used, and the best representative hormone-dependent transformed phenotype.

The effectiveness of the psoralen compounds has been attributed to their DNA photobinding properties, thereby preventing cellular division [6–10].

The B treatment affects the PI3K/AKT survival signal in MCF-7 cells and, furthermore, the same results were established in IGF-I – stimulated cells, where the drug was also active in antagonizing the up-regulatory effect of this growth factor on phospho-AKT protein. The PUVA treatment, in this last experimental condition, does not give any additional effect. The last data acquire particular relevance considering previous findings which demonstrated the crucial role of IGF-I and/or estradiol in maintaining breast cancer cell growth by influencing the PI3K pathways [15–17].

On the other hand, B has elicited an enhanced expression of p53 mRNA and protein content and it has also enhanced the p53 nuclear localization. In this sub-cellular compartment p53 acts as a factor involved in transcriptional regulation of two well known p53 target genes with opposite functions like Bcl-2 and PTEN [18,19].

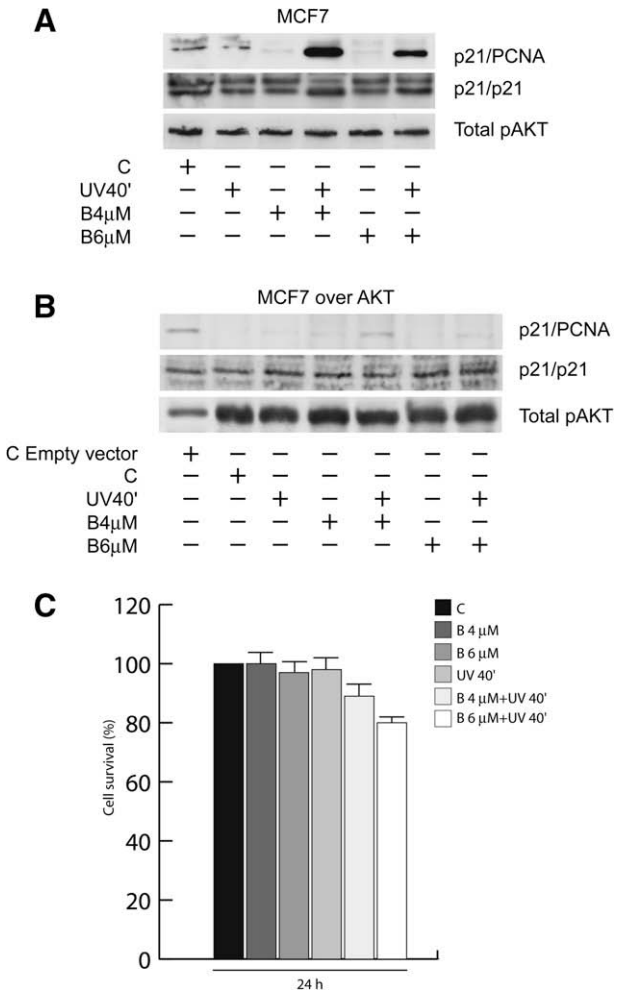

Fig. 3. Different expression of p21-PCNA complex in MCF-7 wild type and MCF-7 cells over expressing AKT protein, treated with bergapten and PUVA. Influence on cell proliferation. Cellular extracts were prepared from MCF-7 cells (A) and MCF-7 cells transient transfected with plasmid pcDNA3-c-Akt encoding for AKT protein (B), treated for 24 h with bergapten (B) and UVA as shown. Immunoprecipitation assay was performed using anti-p21 waf antibody. Immunoprecipitated proteins were resolved and subjected to immunoblotting with anti-PCNA antibody and anti-p21 waf. Prior the immunoprecipitation experiment an aliquot of the lysate corresponding to 20 μg of protein was loaded to determined total pAKT levels in MCF-7 wild type and MCF-7 cells overexpressing p-AKT. C: untreated control cells; C Empty vector: untreated cells transfected with plasmid pcDNA3 empty vector. Results are representative of three independent experiments. (C) MCF-7 cells transient transfected with plasmid pcDNA3-c-Akt encoding for AKT protein were subject to bergapten and bergapten + UV (40 min) treatment for 24 h. The values are expressed as percentage of the control, determined by standardizing untreated cells to 100%. C: control; B: bergapten. Triplicate results are expressed as mean ± S.D. (n = 3).

The down-regulation of anti-apoptotic Bcl-2 and the enhanced expression of p53, obtained in MCF-7 cells under B treatment, well fit with previous findings concerning the direct binding of the oncosoppressor protein to the negative responsive-element present in the promoter region of Bcl-2 gene [18]. The down-regulation of Bcl-2 may result in an increase of apoptosis, as we observed under PUVA treatments, and it is consistent with the cleavage of caspase-9.

In addition, the main feature of apoptosis in MCF-7 cells, undergone to PUVA treatment, has been the formation of 180–200-bp segments, which resulted from the double-stranded nucleosomal DNA fragmentation. These data emphasize how low doses of the photoactivated product have remarkable effects on tumoral cell proliferation and on apoptotic signals. Whereas, in the absence of UV light, higher doses of the drug are necessary to reproduce analogous effects, as documented in our previous paper [13].

The low phospho-AKT levels, induced by the treatment, affect the MCF-7 cell survival. Under this condition, p21, associated with PCNA, produced MCF-7 cell proliferation arrest. On the contrary, this event was completely abrogated when we induced AKT protein over expression. Indeed, MCF-7 cells over expressing AKT were less responsive to psoralen and UV, with respect to MCF-7 wild type. In the first cell line reported, the release of PCNA from complex with p21 contributed to maintain DNA replication.

In conclusion, we have found that psoralen, and in a higher extent the photoactivated molecule, can significantly affect breast cancer cell proliferations. Furthermore, a dual action of psoralen was observed in MCF-7 cells: B is able to significantly reduce the p-AKT survival signalling, while the photoactivated B induced apoptosis, as revealed by the increase of p53, caspase activation and DNA ladder.

Our studies suggest that B alone, or as a photoactivated product, could be used as an active molecule able to counteract effectively the survival and growth of breast hormone-responsive tumors.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2010.04.001.

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